Effect of endophytic bacteria *Bhurkholderia cepacia* on growth, cocoon characters and enzyme activity of *silkworm, Bombyx mori* L.

Abstract:

SILK-The queen of textiles, is the natural fiber, spells luxury, elegance, class and comfort, which is secreted by silkworm. India is the second largest consumer of silk in the world. Silk worm has various advantages as experimental animal such as the low cost for rearing and fewer ethical issues (Yasuhiko Matsumoto et al., 2019). The highly intensive mulberry cropping system causes depletion of nutrients in soil and excess usage of inorganic fertilizers and pesticides caused deleterious effect on soil health and also leaf quality. The quality of leaf fed to the worms is considered to be the prime factor for good cocoon crop production. Better the quality of leaves, greater would be the possibilities of getting good cocoon harvest. Hence, nutrition of silkworm, *Bombyx mori L.* is of primary importance as the cocoon production is directly influenced by the quality of mulberry leaves.Generally, at the first larvae development high temperaure prolong life span and determine cocoon character. However, the fluctuation and wide range temperature inhibit larvae development (MasittaTanjung et al.,2016). Mulberry (*Morus alba* L.) leaves, being the only source of nourishment is certainly imperative that the supply of good quality leaf is most important for getting good quality cocoons (Shashidran et al., 2004). Mulberry harbors a large number of microbes which include bacteria like *Burkholderiacepacia, Bacillus subtilis, Pseudomonas aeruginosa*etc and fungi, actinomycetes. Among which *B. cepacia, B. subtilis*and *P. aeruginosa* have been reported to be endophytic bacteria of mulberry which improves plant growth and control of foliar and soil borne fungal and bacterial pathogens ofmulberry. There exist a mutualistic association between a number of insect species and their extracellular gut micro flora, and they contribute to the nutrition of the host. An indigenous biota is present in all the individuals of a species and maintains stable climax communities in the intestinal mileu of the species.

Few endophytic bacteria were proved to be effective in control of root rot of mulberry (Gunasekhar et al., 2011) and endophytic in nature, when applied to soil it reaches to leaf within short period. The endophytic bacteria (*B.subtilis, B.cepacia* and *P. aeruginosa*) are reported to produce plant growth hormones, solubilize phosphates, fix nitrogen and produce siderophores in plants. For silkworm growth and development, amylase and protease activity in the gut region play much role for digestion and growth of larvae. In the present study, an attempt has been made to know the probiotic/deleterious effect of *Burkholderia cepacia* on silkworm growth and development and the effect on amylase and protease activity in silkworm in midgut tissue and midgut juice. This is informative to know the probiotic activity of bacteria in silkworm which improves larval growth.

Review of Literature:

Mulberry *(Morus alba* L.) the only food crop for silkworm is widely cultivated throughout subtropical, tropical and temperate regions in the world. In India, mulberry is cultivated in 2 lakh hectares under different agro climatic conditions. The sustainable leaf production, silkworm rearing and cocoon production is dependent on soil fertility of mulberry garden. Mulberry is infected by a number of root diseases among them root rot disease of mulberry caused by *Rhizoctonia bataticola* poses a serious threat in all mulberry growing areas throughout the year leading to the death of plants within a short period (Philip et al., 1997, Chowdary et al., 2003). Integrated methods used for control of diseases especially root rot is still posing threats to mulberry cultivation. Besides pathogens, it also contains a group of beneficial microbe viz nitrogen fixers, phosphate solubilizers, potassium solubilizers and antagonistic bacteria /fungi. Among them few endophytic bacteria were proved to be effective in control of root rot of mulberry (Gunasekar et al., 2011) and also improve plant growth (Xianling et al., 2010).

Silk is one of the nature's gifts to mankind produced by silkworm. Sericulture is a highly remunerative agro based enterprise. India is the unique country in the world to produce all the four types of commercial silk and stands second in the production of mulberry silk(Murugesh et al.,2004). However, disease resistance and other improved traits which can augment productivity and quality are needed to be inoculated to enhance the economic benefits to the sericulture farmers (Nagaraju, 2002). Mulberry leaf, being the only source of nourishment is certainly imperative that the supply of good quality leaf is most important for getting good quality cocoons (Shashindran et al., 2004). The mature instar larvae feed on mature leaves that are rich in carbohydrates with lower amount of protein and water content (Aruga, 1994). Thefoliageleaves are the most conspicuous organ of a plant. The structural component of leaf is composed of cellulose, xylan, pectic substance and lignin (Salisburg and Ross,2011). Mulberry leaves are mainly composed of pectin, xylan, cellulose and starch.

Silkworm (*Bombyx mori L.)* is well known Lepidopteron (Family: Bombycidae) , the larvae instars of which feed on the leaves of mulberry used for silk production. Indian silk industry is based largely on the mulberry silkworm. As the insect for silk production, silk worm has very economic value because silk is a very good textile material and is utilized widely (Yanhua Yang et al., 2018). Economics of silk production depends on the quality of cocoons produced by the worm (Krishna swami and Sundaramullary, 1991), which in turn is depended upon the nutritional demands of silkworm. Considering the monophagous nature of silkworm, depending solely on mulberry leaves, the options available are restricted to the improvement of nutritional quality of leaves, use of nutrient additives and supplementary ingredients which can be routed through mulberry leaves. Recent approaches in this direction include the application of VAM fungi and bacterial biofertilizers to improve the mulberry leaves quality and thereby the cocoon characters (Rao et al., 2007). The fresh and nutritive quality of mulberry leaves plays an important role on the development of worm stabilizing the cocoon production and silk productivity. The various compositional factors of mulberry leaves are responsible for successful cocoon harvest and silk productivity, thus the mulberry leaf quality plays a predominant role in healthy growth of silkworm. Hence, nutrition of silkworm *Bombyx mori L.* is of primary importance as the cocoon production is directly influenced by the nutritive status of mulberry leaves. The quality of feed is determined by its major components such as water, carbohydrates, proteins, minerals, elements,

fats, amino acids and vitamins (Thirumalaiswamy et al., 2009).

The silkworm is considered a central model species for lepidopteran genomics and genetics, and it is second only to the fruit fly (*Drosophila melanogaster*) as an insect model for biological studies (Fang Lu et al., 2020).Mulberry silkworm, *Bombyxmori*is susceptible to a number of diseases and also to the attack of pests and parasites. There is no silkworm race at present, which can be deemed as totally resistant to diseases or pest (Nagaraju, 2002). The fungi, bacteria, nematode and viral diseases persist throughout the year. Though most of these diseases appear and cause maximum damage during rainy and winter season, there are also few diseases that appear during summer and cause reduction in plant growth (Aleksey chenko et al.,2004).

Silkworm is poikilotherm; it cannot regulate its body temperature and is susceptible to several diseases (Prasad, 1999). Diseases in silkworm and mulberry plants caused by pathogens reduce the quality and quantity of silk production which in turn affects normal economy. Attempts have been made in sericulture with nutrient such as protein, vitamin, carbohydrates, amino acids, hormones and antibiotic etc for better performance of good quality of cocoons (Sannapa, 2002).

Gut micro flora is regarded as valuable metabolic resources for the insect on suboptimal diets, but apart from this, most relationship and their micro biota remain undefined. Microbial transformation of plant secondary compounds in an insect gut and adoption by the host to use resulting common metabolites are unique to insects (Dillon, 2000). Some of the gut micro flora of silkworm includes *Bacillus cereus, Bacillus subtilis, Lactococcuslactis, Staphylococcus lactis, Enterobacteraerogenes*etc (Sekar et al., 2010).

Probiotics are the live microbial food supplements beneficially affecting host by improving the microbial balance and enhanced rapid cellular growth anddevelopment(Fuller et al.,1993). The gut probiotics are involved in the digestive utilization of feeds and detoxification of metabolite. Stimulation of non specific immune system. They also promote the production of vitamins and increase host resistance and compete with pathogenic bacteria by producing organic and antibiotic substances. The *Lactobacillus plantarum*is a probiotic which improves the cocoon production of mulberry silkworm *Bombyx mori* (Singh et al., 2005). Certain probiotic bacteria inhibit the growth ofmicrobes.

*Streptomyces nourse*iare probiotic microbes which prove the antibacterial activity and good ecofriendly management of silkworm diseases (Subramanian et a., 2009). Impact of robotics (*Lactobacillus, Saccharomyces cereviciae* and effective microorganisms) treatment on mulberry leaves to modulate the economic parameters of 5thinstars larvae of *Bombyx mori* were studied (Jeyapaul et al., 2004) Amala et al.,2011 had stated that *S. cereviciae* serves as an immune modulating agent in silkworm *Bombyxmori.* When the probiotic *S. cereviciae* was used for the treatment there was a considerable increase on the energy budget and the commercial characteristics of *Bombyx mori* and also there was an increase in the level of protein content in treated worms. Yeast improves the protein content and commercialproduction.

The leaves of mulberry are the sole source of food for larval instars of silkworm *Bombyx mori*, biochemically constituted with proteins, lipids, carbohydrates and minerals. Therefore, corresponding diversity of enzymes capable of hydrolyzing the biocompounds of mulberry is exhibited by mid gut of larval instars of silkworm, *Bombyx mori*. It has been suggested that, there is a functional

difference between the activity of digestionbythedigestivefluidinmidgutandtissueofmidgut.Ithasbeenreportedby

Horie et al., (2010) that, molecular proteins are hydrolyzed into peptides by digestive fluid content and into amino acids with peptidase in the mid gut tissue likewise, the polysaccharides, are digested in the insect gut lumen by digestive fluid and disaccharides and/or trisaccharides get hydrolyzed into their constituent monosaccharide sugars mainly in the guttissue.

The highly specialized proteins are called enzymes. Enzymes are the reaction catalyst of any biological system. The digestibility of silkworm larva depends upon the activity of an enzyme called amylase. Amylase is one of the most important enzyme which helps in digestion of starch in silkworm. It is the key enzyme involved in digestion and carbohydrate metabolism in insect. Of the various enzymes analyzed amylase is well worked out because of its close association with the economic parameters of silkworm (Esaivani et al., 2014). The literature viz Sengupta et al., 1972; Mathavan et al., 1984; Jeyapaul et al., 2003a and 2003b and Sheeba et al., 2007 reveals that the biochemical formulations promote the levels of enzyme activity which ultimately enhances the quality of thetraits.

Endophytic bacteria are those that colonize plant tissue internally without showing any external symptoms or negative effects on their host. Research has shown the potential of endophytic bacteria as biocontrol and plant growth promoting agents. The *Burkholderia cepacia*complex (BCC) is a diverse group of bacteria commonly found in soil, water and the rhizosphere; on bodies of animals including humans; and in the hospital environment. As entophytic bacteria, members of *Burkholderia cepacia* complex have been isolated from a few crops such as sweet corn, cotton, rice, yellow lupine and sugarcane, and *B.cepacia* strains have proved useful as antagonists of plant

pests and in increasing the yield of several crop plants (Xianling et al., 2010). Mulberry anthracnose caused by *Colleto trichumdematium*; is a commonly observed disease and has become a serious threat to the production and quality of mulberry leaves in susceptible varities and thus a major problem in mulberry cultivation in China. As silkworms are reared on mulberry leaves, improper use of agrochemicals to treat the disease could be hazardous to the worms. Therefore, the use of agrochemicals has not gained wide acceptance in mulberry gardens, and the need for alternative techniques that are safe to silkworms is acutely felt. Biological control of plant pathogens using antagonistic bacteria is a promising strategy and has attracted considerable attention in the efforts to reduce the use of agriculturalchemicals.

Strain Lu10-1 of *Burkholderia cetacean* (Gene bank EF 546394) is an antagonistic entophyte originally isolated from mulberry (*Morus alba*) leaves. *B.cepacia* strain Lu10-1 is an endophyte that can multiply and spread in mulberry seedlings rapidly and efficiently. The strain is antagonistic to *C.dematium* and act as an efficient plant growth promoting agent on mulberry seedlings and is therefore a promising candidate as a biocontrol and growth promoting agent (Xianling et al.,2010).

In the present study, an attempt has been made to know the effect of endophytic bacteria *Burkholderiacepacia*on growth and development of silkworm as well as the activity of digestive enzymes i.e., amylase and protease are estimated by standard procedures.

List of gut micro flora of silkworm *(Bombyx mori*):

BACTERIA	REFERENCE
Bacillus cereus	Sekar,P., Balasundaram,A and George John study on the establishment of bacterial micr the gut of silkworm *Bombyx mori*. Internationa of current research.11:192-199.
Bacillus subtilis	
Lactococcuslactis	
Staphylococcus lactis	
Enterobacter aerogenes	
Pseudomonas aeruginosa	Vitthalrao B Khyade and Rjendra M Marat Diversity of bacterial flora in the midget of fi larvae of silkworm, *Bombyx mori* (race:PM×CSR2).G.J.B.B.1:191-200.
Bacillus circulans	
Proteus vulgaris	
Klebsiella pneumonia	
Escherichia coli	
Citrobacter freundii	
Serratia liquifaciens	
Enterobacter sp.	
Pseudomonas fluorescens	
Aeromonas sp.	
Erwiniasp.	
Streptomyces noursei	Mohanraj P and Subramanian S.2014. Ant activity of gut flora isolates from mulberry s *Bombyx mori*. An international quarterly jo environmental sciences(special issue).1:267-270
Bacillus subtilis	

Materials and Method: To study the effect of endophytic bacteria, *Burkholderia cepacia*(Rifampicine resistant bacterial strain) was collected from the stock culture of Agronomy section, CSRTI, Mysuru. Silkworm weight and other statistical data were collected from CSRTI rearing section,Mysuru,Karnataka,India.

MATERIALS REQIURED:

Starch solution (1%), DNS reagent, Phosphate buffer (pH 6.8), Casein solution (1%), Ninhydrin reagent and Sucrose solution 0.25M.

INOCULUM PREPARATION:

Burkholderia cepacia culture was multiplied on LB agar media. Streaked bacteria on LB agar plate were incubated at 30+-2^{o}c for 24 hours. A loopful of 24 hour bacterial culture, *Burkholderia cepacia* was inoculated to 250 ml conical flask containing LB broth. The inoculated cultures were incubated at 30+-2^{0}c at 100 rpm in an orbital shaking incubator (Paragon RPM-0249). 48 hours old bacterial culture was then centrifuged at 12000 rpm for 30 minute (Refrigerated centrifuge REMI CH 12). The supernatant was discarded and the pellets obtained were dissolved in 100ml physiological saline water. Bacterial cell concentration was adjusted to 10^{8} CFU/ml (by adding physiological saline water) with the help of UV or visible spectrophotometer (ELICO SL 171 mini spec) at 660nm (Optical density for 10^{8} CFU/ml at 0.1). From 10^{8} CFU/ml concentrations, 10^{6} CFU/ml suspensions were prepared using serial dilution method.

BIOASSAY:

A popular silkworm double hybrid (CSR50×CSR52) × (CSR51×CSR53) was used for the bioassay experiments. The layings were obtained from silkworm seed production centre, Mysore and the experiments were conducted at silkworm physiology laboratory, CSRTI, Mysore. The hatched larvae are reared in plastic trays as per standard procedures. After fourth moult, the larvae were used for experimentation. 100 healthy larvae of 5thstage (before 1stfeeding) were selected and kept in plastic trays. For each treatment, 3 replicates were maintained.

INOCULATION OF BACTERIA TO SILKWORM:

For 1stfeeding of 5thstage larvae, *Burkholderia cepacia* suspensions were injected orally by feeding through mulberry leaves. Two concentrations of bacteria 10^{6} and 10^{8} CFU/ml was prepared as described earlier. Healthy mulberry leaves were cut into 10cm discs, 5 such discs were fed to 100 larvae of silkworm. Before feeding, 1ml of inoculums was evenly spread on the dorsal side of the leaf disc with sterile plastic spreader. 2 treatments 10^{6} and 10^{8} CFU/ml were tested. For control 5 such discs were treated with 1ml of physiological saline water. 2ndfeeding onwards normal leaves were

fed up to the spinning. For each treatment 3 replicates were maintained.

DATA ON LARVALGROWTH:

During 5th stage of larvae, before first feeding 10 larval weights were recorded. 3 replicates were maintained for each treatment and control. During 5th stage of larvae, the data on larval weight was recorded at 24 hours interval up to 6 days i.e., up to the maturity of worms forspinning.

AMYLASE AND PROTEASE ACTIVITY:

Amylase and protease activity in silkworm gut juice and tissue was estimated on 5th day larvae of 5th instar. Mid gut tissue and mid gut juice were collected for the estimation of amylase and proteaseactivity.

ISOLATION OF MIDGUT JUICE AND MIDGUT TISSUE:

0.5 ml of mid gut juice was drawn from the anterior end of silkworm 5th day of 5th instar larvae in an eppendroff's tube rinsed with an anticoagulant Thiourea.

Similarly mid gut tissue was excised by cutting larval skin dorsally in a dissection tray containing ice cold ringer solution with TrisHCl buffer (pH 7). Mid gut tissue was collected by separating anterior and posterior part of the gut and transferred to a pre cooled plastic vials.

ENZYME ASSAY:

0.1gram of mid gut tissue was collected and ground with 5 ml of 0.25 M sucrose solution in a mortar and pestle. 0.5 ml of mid gut juice and 5 ml of sucrose was mixed. Then the suspensions were centrifuged at 4000 rpm for 30 minutes. 0.5 ml of supernatant from both tissue and juice samples were collected separately in respectively labeled test tubes. 2 ml of phosphate buffer pH(6.8) was added to each test tubes including control. Then 1 ml of 1% starch solution was added to test tubes meant for amylase activity and 1% casein solution was added to test tubes meant for proteaseactivity.Thetesttubeswereincubatedatroomtemperaturefor15minutes.
Then 2 ml of DNS reagent and Ninhydrin reagents were added to amylase and protease test tubes respectively.

The test tubes were kept for water bath for 30 minutes. After cooling, enzyme activity was measured at 540nm spectrophotometrically.

$$\text{Amylase activity} = \frac{\text{Concentration of product formed} \times 2}{\text{Molecular weight of glucose} \times \text{time of incubation}}$$

$$\text{Protease activity} = \frac{\text{Concentration of product formed} \times 2}{\text{Molecular weight of tyrosine} \times \text{time of incubation}}$$

Results and Discussion:

The results obtained were very transparent and have substantiated our research work. The research work has indeed considered several facets ranging from weight to enzyme activity and larval gut microbial profile. Table 1 depicts the outcome of Burkholderia cepacia on the larval growth which has confirmed an upsurge in larval weight. The larval weight was kept under constant observation and the weight was recorded from the 1st day of 5th in-star at intervals of 24 hours for 5 days. The product of the research work has demonstrated an increase in the weight in succession since day 1. The research has also substantiated the relation between the larvae culture and the extent of bacterial load. It was found that the larvae weight enhancement was directly proportional to the extent of bacterial culture. Higher concentrations of bacterial cultures have indeed had an affirmative impact and have positively contributed to larvae weight. The increase in the larval weight was in correspondence to amylase and protease activities measured on 5th day larvae and have been depicted in table 2 and 3. Control was used in accordance to the treated sample in order to decipher the outcome for productive interpretation. Similarly the amylase activity of mid gut juice was recorded as 0.0272µmoles/min/ml, 0.0278µmoles/min/ml and 0.0279µmoles/min/ml in control, T1 and T2 respectively. These results validated the fundamental shift in microbial profile in silkworm larval gut which is beneficial to the host and in turn may significantly contribute to increase silk production. Food has also been a vital criteria in deciding the amount of silk production and has been regulated by its physical nature and presence of phagostimulants in the food. (Dadd, 1970). Silkworm Bombyx mori (L) reared on mulberry leaves supplemented with minerals, oral extracts, plant growth hormones (Sunder Raj et al., 2000) are reported to have beneficial effects on economic parameters.

The increase in the larval and cocoon weight was in correspondence of protease activity measured on 5th day larvae. Protease activity in tissue was observed to be 0.042µmoles/min/ml of sample in control and 0.070µmoles/min/ml and 0.082µmoles/min/ml in T1 and T2 respectively. Similarly the protease activity in mid gut juice was observed to be 0.178µmoles/min/ml in control, 0.296µmoles/min/ml in T1 and 0.334µmoles/min/ml in T2 respectively. Burkholderia cepacia was fed to silkworm orally through mulberry leaf, it reaches to mid gut and survive for life time and increases the enzyme activity of silkworm and improves its digestivity(Figure 4).Experimental results on the isolation of Burkholderia cepacia fed to the silkworm from the fecal matter after ingestion to the larvae revealed that the bacteria survived in the digestive tract. Similarly as amylase and protease activity also represented in table 2 and 3, the results indicated that the amylase and protease activity of 5thinstar larvae mainly for the digestion and absorption of sugar and protein content of the mulberry leaves consequently which increases haemolymph and silk gland protein content ultimately increases silk productivity of the silkworm. (Thirumalaisamy R et al.,2009). Glycogen being a storage polysaccharide was found to be high in the experimental groups of silkworm *Bombyx mori*. It is significant to correlate to the availability of increased sugars, which may undergo glycogenesis resulting in more amount of glycogen. Amylase catalyses the specific hydrolysis of the glycosidic bonds in specific hydrolysis of the glycosidic bonds in glycogen (Plummer, 1988).Hence theincreased amount of glycogen may bring about the increased secretion of digestive enzyme amylase. Increase in protease activity may be attributed to the increased concentration of silk protein for silk production. The digestive tissue may be tuned to synthesize more of protease enzyme since the protein content increased significantly over control category on UV ray treatment at 280-400nm (Mohamed Sadiq A et al.,2008). The results on cocoon characters were presented in table 4. Single cocoon weight , Single shell weight and SR% was increased in both treatments. The SR% in control was 21.27 and T1 and T2 was 21.64 and 22.76 respectively. The SR% may be in correspondence with amylase and protease activity of silkworm larvae treated with 10^6 (T1) and 10^8 (T2) concentrations of *Burkholderia cepacia*.

Tables and Graphs:

Treatment	Grams				
	1st day	2nd day	3rd day	4th day	5th day
C	14.079	20.458	31.474	40.909	44.403
T1	14.391	23.328	33.324	43.79	45.478
T2	16.381	25.013	34.095	44.838	46.459

Table 1: Represents average weight of 10 larvae of 5th instar from 1st to 5th day.

Treatment	µmoles/min/ml of sample	
	Midgut tissue	Midgut juice
C	0.0346	0.0272
T1	0.0359	0.0278
T2	0.0376	0.0279

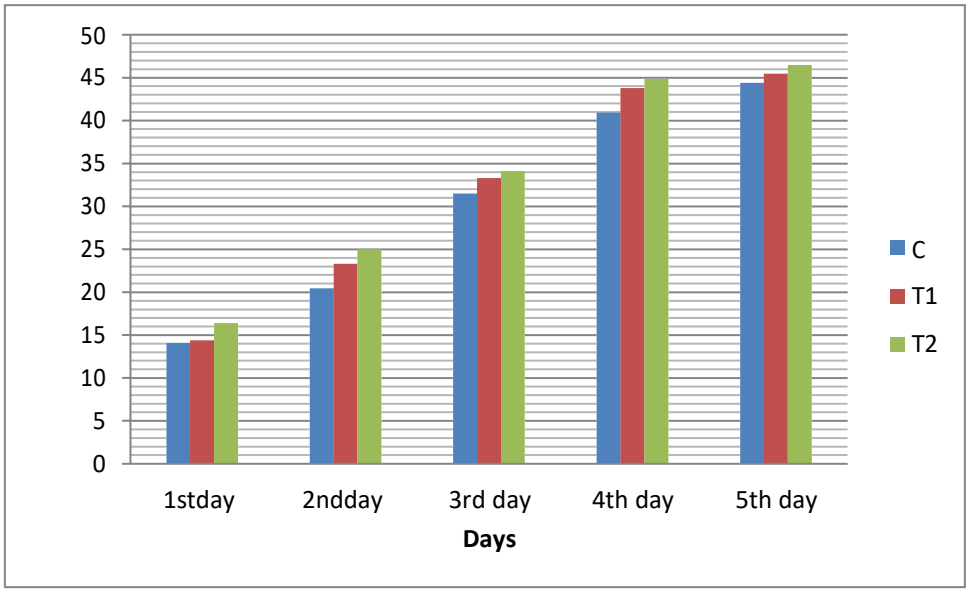
Table 2: Represents amylase activity of 5th day larvae of 5th instar

Treatment	µmoles/min/ml of sample	
	Midgut tissue	Midgut juice
C	0.042	0.178
T1	0.070	0.296
T2	0.082	0.334

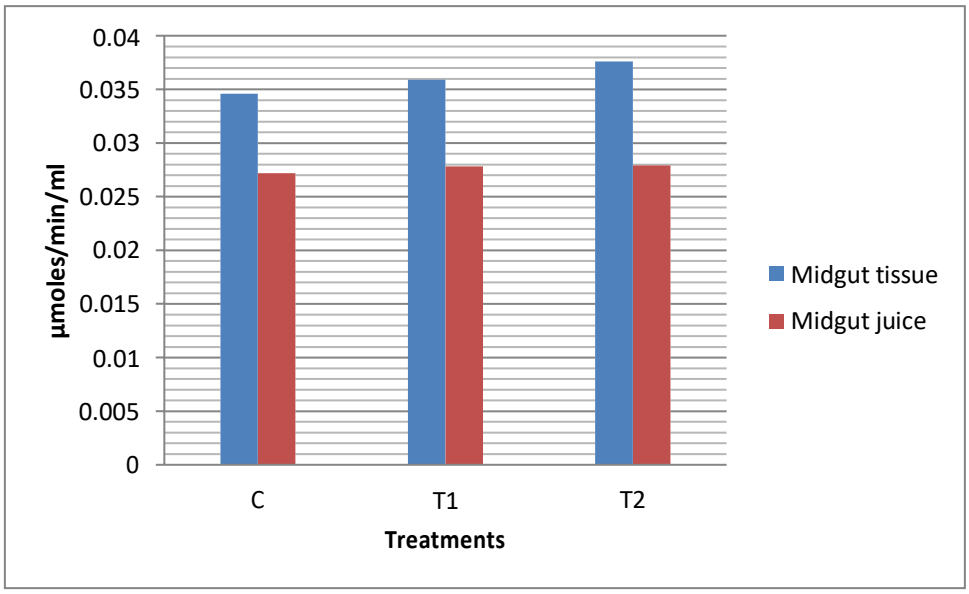
Table 3: Represents protease activity of 5th day larvae of 5th instar

	SCW	SSW	SR%
C	2.096	0.446	21.27
T1	2.121	0.459	21.64
T2	2.051	0.467	22.76

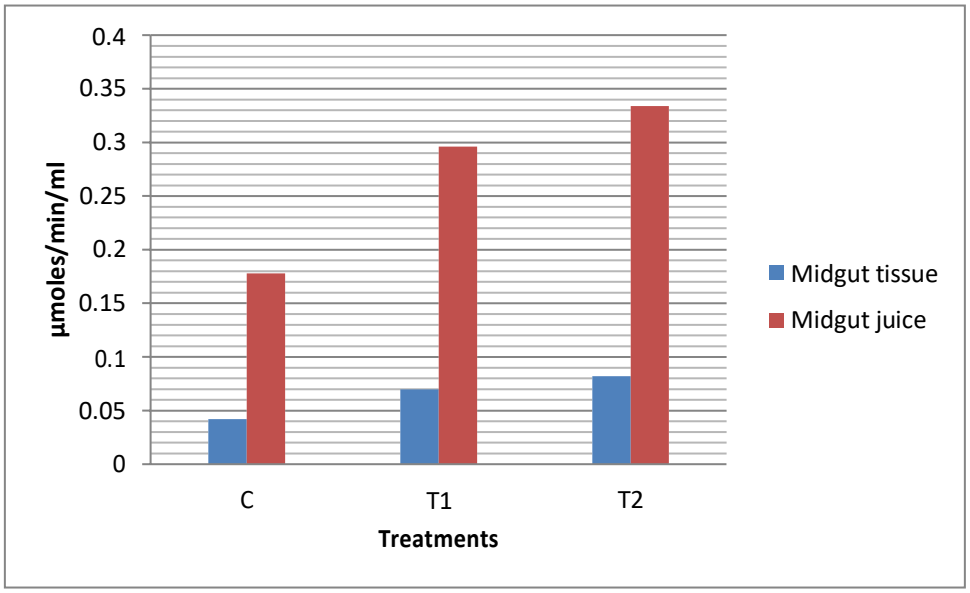
Table 4: Represents results on cocoon characters. SCW-single cocoon weight , SSW- single shell weight and SR%-shell ratio.



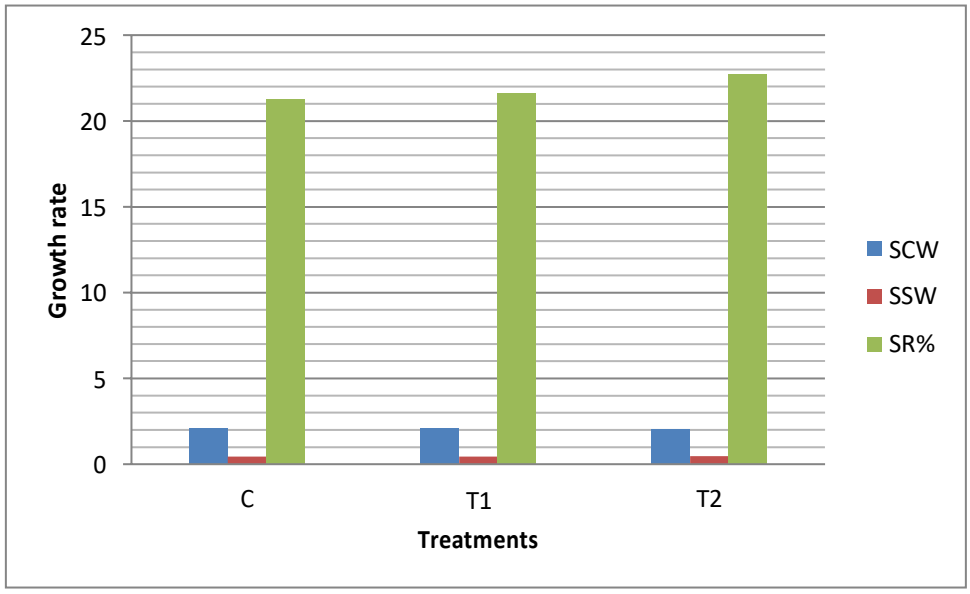
Graph 1: Average weight of 10 larvae of 5thinstar from 1stto 5thday.



Graph 2: Amylase activity of 5thday larvae of 5thinstar.



Graph 3: Protease activity of 5thday larvae of 5thinstar.



Graph 4: Cocoon characters-single cocoon weight,single shell weight and shell ratio.

Photos:

Figure 1: Pure cultures of Burkholderia cepacia collected from stock culture of Agronomy section, CSRTI, Mysore.

Figure 2: Luria bertani broth containing cultures of Burkholderia cepacia

Figure 3(a): Dorsal side of mulberry leaves spread with inoculum were fed to 1stday of 5thinstar larvae

Figure 3(b): 2ndday of 5thinstar larvae fed wuth normal leaves

Figure 3(c): 3rdday larvae of 5thinstar

Figure 3(d): 4thday larvae of 5thinstar

Figure 3(e): 5thday larvae of 5thinstar

Figure 3(f): Zoomed image of 5thday larvae of 5thinstar

Figure 4: Isolation of Burkholderia cepacia from the fecal matter of silkworm fed with bacterial inoculum

Figure 5: Spinning of silkworms on collapsible plastic mountages

Figure 6: Formation of cocoons by silkworms

Figure 7: Cocoons formed after complete spinning of silkworms

Figure 8: Pupa and shells collected for assessment

Figure 9(a): Male pupae

Figure 9 (b): Female pupae

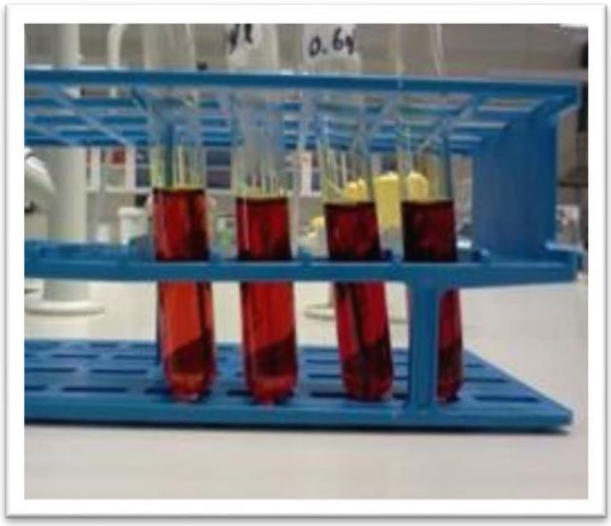


Figure 10: Estimation of amylase activity of silkworm midgut tissue and midgut juice samples

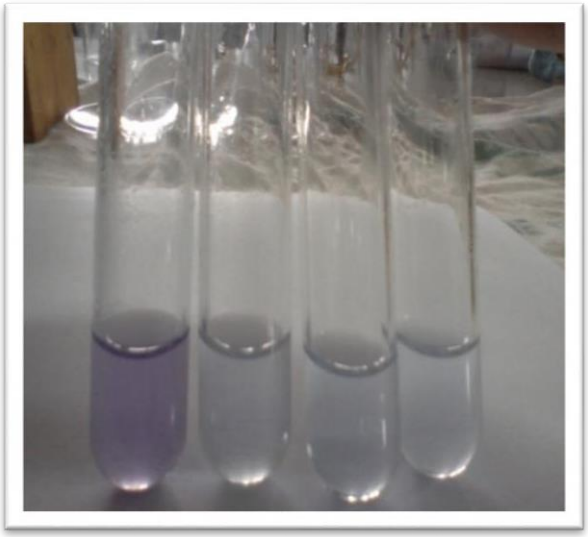


Figure 11: Estimation of protease activity of silkworm midgut tissue and midgutjuice samples

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